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Bureau of Agricultural and Industrial Chemistry

PREPARATION OF CHLOROPHYLL DERIVATIVES FOR INDUSTRIAL AND PHARMACEUTICAL USE

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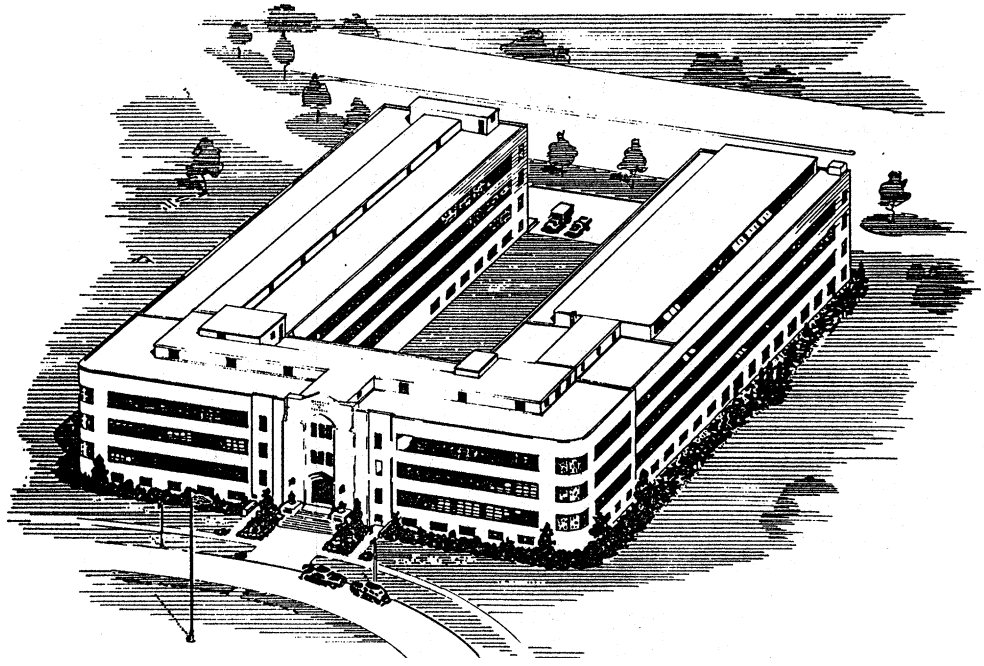

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Within the last few years interest in chlorophyll, and particularly in its water-soluble copper derivative, sodium copper chlorophyllin, has increased markedly. The latter compound has been used in proprietary products for removing objectionable odors from rooms (4). A recent report by Moss et al. (3) showed that an ointment containing water-soluble chlorophyll had excellent deodorant powers for suppurating wounds and ulcers. The compound has also been recommended for control of body and breath odors (5, 10), a usage which may provide a large market for chlorophyll derivatives.

This Laboratory has recently received many requests for information on the extraction and preparation of chlorophyll and its derivatives. In an effort to meet these requests, results of research carried out at the Eastern Regional Research Laboratory are here made available.

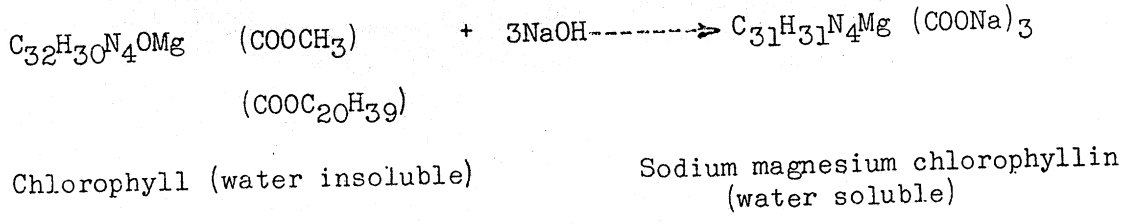
Chemistry of the Process

The chlorophyll derivative most frequently used at present is the water-soluble sodium copper chlorophyllin. The process to be described involves the preparation of this compound from chlorophyll in plant extracts.

Chlorophyll exists in higher plants in two closely related forms, designated "a" and "b". The a form is the predominant constituent by a rather constant 2:1 ratio. For the sake of convenience, we shall consider that all chlorophyll is in this form.

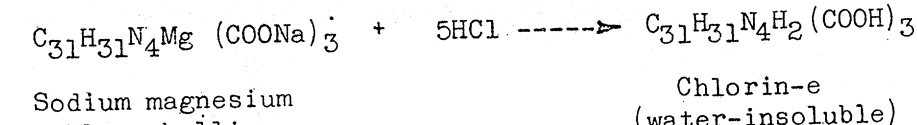
The four following reactions are involved in the conversion of chlorophyll to sodium copper chlorophyllin.

1. Alkaline hydrolysis of the methyl and phytyl esters and opening of an internal oxygen ring. The end product is sodium magnesium chlorophyllin.



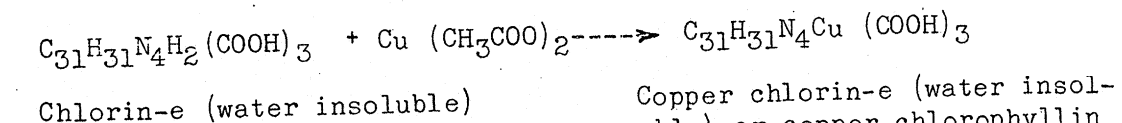
Chlorophyll (water insoluble) — Sodium magnesium chlorophyllin (water soluble)

2. Replacement of magnesium and sodium by hydrogen.



Sodium magnesium chlorophyllin (water soluble) — Chlorin-e (water-insoluble)

3. Replacement of hydrogen by copper.



Chlorin-e (water insoluble) — Copper chlorin-e (water insoluble) or copper chlorophyllin

4. Formation of sodium salt

$$C_{31}H_{31}N_4Cu(COOH)_3 + 3NaOH \longrightarrow C_{31}H_{31}N_4Cu(COONa)_3$$

Copper chlorin-e (water insoluble) — Sodium copper chlorin-e or sodium copper chlorophyllin (water soluble)

For further information, the reader is referred to the classical and fundamental investigations of R. Willstätter (11).

The following flow sheet and text give the detailed procedure for preparation of sodium copper chlorophyllin. Chlorophyll salts containing iron, zinc, and other metals can be prepared by similar procedures.

Flow sheet for preparation of sodium copper chlorophyllin from chlorophyll extracts

Broccoli leaf meal, 20 pounds (approximately 0.8% chlorophyll)
Extract with acetone, 5 gal.

- Extract containing chlorophyll, 50-70 grams — Residual meal
- Concentrate to dryness
- Saponify with 1 liter methanol saturated with NaOH
- Extract saponification mixture with hexane
- Aqueous solution containing sodium magnesium chlorophyllin — Hexane solution containing unsaponifiable matter
- Acidify with HCl to pH 5.0
- Extract with hexane
- Decant and filter
- Chlorin-e precipitate, 35 - 40 grams — Filtrate discard
- Dissolve in boiling acetic acid, 200-300 ml.
- Add 10% copper acetate solution, 500 ml.
- Filter
- Copper chlorin-e precipitate 40-45 grams — Filtrate discard
- Suspend in 50% aqueous ethanol, 400-500 ml.
- Add sodium hydroxide, 0.18 gram per gram copper derivative
- Evaporate to dryness
- Sodium copper chlorophyllin, 40-45 grams, (dry, water-soluble, powder).

Sources of chlorophyll

Chlorophyll is most conveniently extracted from dehydrated leaf meals. Alfalfa, the most readily available source, has a chlorophyll content of 0.2 to 0.4 per cent. Broccoli leaf meal, available in limited quantities, has a much higher chlorophyll content, which in many cases ranges from 0.8 to 1.0 per cent.

Extraction

The following procedures are based on use of 20 pounds of broccoli leaf meal. The meal, which should not be ground finer than 20 mesh, is placed in a large Soxhlet extractor and continuously extracted with 5 gallons of acetone. Under these conditions, 80-85 per cent of the total chlorophyll is extracted in 4 to 8 hours.

Chlorophyll can also be extracted from leaf meal with ethanol or isopropyl alcohol. All the solvents mentioned are more effective for extraction when they contain 5 to 20 per cent water. It is difficult, however, to concentrate aqueous extracts, and therefore we have used anhydrous acetone. In typical experiments, extracts containing 50 to 70 grams of chlorophyll were obtained.

Preparation of sodium magnesium chlorophyllin

The acetone extract is now concentrated almost to dryness. Most of the acetone is evaporated at atmospheric pressure, but the last 1/2 - 1 gallon portions are best removed under vacuum. One to two liters of methanol saturated with sodium hydroxide (about 10%) is brought to a boil and mixed with the chlorophyll concentrate. The resultant mixture is refluxed for 10 to 15 minutes, cooled, and diluted with water to make a total volume of 6 liters.

At this stage of the procedure, the saponified extract contains the chlorophyll salt, sodium soaps from the saponification of plant glycerides, and much unsaponifiable matter, including phytol, carotene, tocopherol and sterols. To purify the extract, it is necessary to remove the unsaponifiable matter. This is best accomplished by placing the extract in a column packed with Raschig rings and continuously circulating hexane through the aqueous liquid. A 2- to 3-hour extraction period is quite adequate. The hexane is recovered, and some of the valuable unsaponifiable constituents may be recovered, if desired, by methods developed at this Laboratory (8, 9).

Preparation of Chlorin-e

The aqueous alkaline extract remaining after the hexane extraction is brought to a pH of approximately 5.0 by careful addition of hydrochloric acid. Under these conditions, chlorin-e and free fatty acids derived from saponified glycerides are precipitated. This mixture is difficult to filter unless the fatty acids are removed. Chlorin-e is insoluble in both

water and hexane; the fatty acids are hexane soluble and hence can be easily separated from the chlorin-e. The aqueous chlorin-e suspension is therefore shaken or stirred with several liters of hexane, and the hexane layer is drawn off by suction. The chlorin-e is lighter than water and floats at the surface. Most of the bottom water layer can be drawn off by suction.

The chlorin-e is filtered on a Buchner funnel, washed with hexane and then with slightly acidified water to prevent the chlorin-e from forming a colloidal suspension, which is virtually unfilterable. The chlorin-e precipitate is finally dried by suction, and stored in closed containers. It should not be dried by heating, except small samples for moisture determination. Heating converts chlorin-e into an insoluble form, from which it is difficult to prepare water-soluble derivatives.

On account of its insolubility in both water and hexane, chlorin-e can be prepared in quantitative yield and with a purity of 85 to 95 per cent. In our experiments, extracts containing 50 to 70 grams of chlorophyll yielded 35 to 45 grams of chlorin-e, moisture-free basis.

Preparation of copper chlorophyllin

The chlorin-e preparation, obtained as previously described, is dissolved in 200 to 300 ml. of boiling glacial acetic acid. To this solution, 500 ml. of a boiling 10 per cent aqueous solution of cupric acetate is slowly added. The mixture is boiled for 5 to 10 minutes, allowed to cool, and diluted with water, and the insoluble copper chlorophyllin is filtered. The precipitate is thoroughly washed with hot, slightly acidified water, and then dried by suction. It should not be dried by heating.

The yields are 90 to 95 per cent and the purity 90 per cent or better, based on copper content. Forty to fifty grams of copper derivative, moisture-free basis, was obtained from 35 to 40 gram batches of chlorin-e. This derivative is oil soluble and should be used for purposes that require oil solubility and water insolubility.

Copper chlorophyllin can also be made by introducing copper directly into the chlorophyll molecule before isolation of chlorin-e. The hexane-extracted aqueous alkaline solution is brought to pH 8.0 by careful treatment with hydrochloric acid, and stirred with 500 ml. of 10 per cent aqueous copper acetate, after which the pH is lowered to 4.0 by further addition of acid. The procedure from this point is identical with that described under chlorin-e after acidification. Since many other compounds capable of forming insoluble copper salts are present in crude plant extracts, this method is not recommended for preparing high-grade copper chlorophyllin.

Preparation of sodium copper chlorophyllin

The copper chlorophyllin is suspended in 50 per cent aqueous ethanol (10-20 ml. per gram of derivative, moisture-free basis). It is refluxed for one-half hour with an equal volume of 50 per cent aqueous ethanol containing

the theoretical quantity of sodium hydroxide required for reaction with the copper derivative. For every gram of 100 per cent copper chlorophyllin, moisture-free basis, 0.18 gram of sodium hydroxide is required. Since it is difficult to remove excess alkali, the purity of the copper compound should be carefully determined and appropriate adjustments of the quantity of alkali made if necessary. For example, if the copper derivative is 90 per cent pure, then 0.18 x 0.90 = 0.16 gram sodium hydroxide would be required for every gram of derivative.

The resultant solution is filtered, any insoluble material is washed with 50 per cent aqueous ethanol, and the filtrate is concentrated to dryness on a steam bath. Yields based on the copper chlorophyllin are almost quantitative. The purity of the product based on copper analysis ranges from 80 to 90 per cent. It should be remembered that sodium copper chlorophyllin is a mixture derived from chlorophylls a and b and hence is not a pure compound.

Methods of Analysis

Preparation of relatively pure sodium copper chlorophyllin requires careful analytical control of a number of steps. A brief description of the required analyses follows:

MOISTURE: Since the chlorin-e and copper chlorophyllin preparations cannot be heat dried, they will have a variable water and organic solvent content. The conversion of chlorin-e to copper chlorophyllin and of copper chlorophyllin to sodium copper chlorophyllin is based on weights calculated to a moisture-free basis. Hence every new batch of these compounds must have an analytical moisture determination. This is simply done by weighing accurately 1.0 to 2.0 grams of chlorophyll derivative and drying to constant weight in a vacuum oven at 60-70° C.

COPPER: The copper content is one of the criteria of the purity of copper chlorophyllin and sodium copper chlorophyllin. Theoretically, the former compound contains 9.65 per cent copper and the latter 8.75 per cent. In most cases, the analysis is unreliable if used alone, because it does not distinguish between organically bound and inorganic copper. It is best used in conjunction with spectrophotometric analysis, which will be described later.

A sample of approximately 0.1 gram (moisture-free basis) is digested with 3 ml. of concentrated sulfuric acid in semi-micro Kjeldahl tubes. When all frothing and foaming ceases and the color of the solution turns black to brown or light brown (this process takes 1 to 2 hours), the solution is cooled. Ten to fifteen drops of 35 per cent perchloric acid is added, and the solution is boiled until it turns colorless or light blue (5 to 10 minutes). This solution is cooled, and diluted to 100 ml., and an aliquot taken for analysis. At this laboratory, a colorimetric method involving formation of a yellow copper diethyl dithiocarbamate is used. Further details can be found in the literature (2, 6, 7).

W-a-r-n-i-n-g. Use of perchloric acid is a dangerous procedure - explosions may occur if the reagent is improperly used. Only qualified and experienced personnel should work with this reagent.

Chlorophyll and its derivatives

A good spectrophotometer is required for work of this type. Chlorophyll in acetone extracts is determined by the method of Comar (1).

No methods are available in the literature for the spectrophotometric determination of sodium copper chlorophyllin. At this laboratory, the specific absorption constant for a fairly pure sodium copper chlorophyllin preparation has been determined. This value is 16.2, solvent 50 per cent aqueous ethanol, wave length 630 mμ. From the relation $a = \frac{D}{Cl}$ where a = specific absorption constant, D = optical density, C = concentration in grams per liter, and l = cell length in centimeters, the concentration of unknown sodium copper chlorophyll solutions can be determined. If the cell length is 1 centimeter and the volume 100 ml., then concentration (mg. per 100 ml.)

$$\frac{D \times 100}{16.2}.$$

Results determined in this manner are intrinsically more reliable than copper determinations because the optical density of a solution of sodium copper chlorophyllin is a direct function of its chlorophyll content, whereas copper may be organically combined with other compounds or be present in uncombined inorganic form.

Acknowledgment

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